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Visualization of the distribution of multiple constituents in bread dough by use of Fluorescence Fingerprint Imaging

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Abstract

A method combining fluorescence fingerprint (FF) measurement with an imaging technique was developed to visualize the distributions of gluten and starch in dough. The fluorescence images of dough at the under-, optimum- and over-mixing stages were taken with a charged coupled device (CCD) camera in multiple excitation and emission wavelengths constructing the FF. This data containing the FF data of each pixel in the sample image was colored according to the similarity of the FF of each pixel and the FF of gluten or starch. Furthermore, the areas corresponding to air bubbles in the dough were masked in black. The resulting image, referred to as the FF pseudocolor image, showed the distributions of gluten and starch in the dough with the incorporated bubbles. Quantitative parameters concerning gluten and starch distributions and bubble area were extracted from the dough images at each mixing stage. It was shown that in the under-mixing stage, the distributions of gluten and starch were more uneven and the bubbles were smaller than those in the optimum-mixing stage. The evenness of gluten and starch distributions stayed nearly the same from the optimum- to over-mixing stages but the total bubble area increased significantly. This imaging method is suggested to have an advantage over conventional imaging methods such as light or electron microscopy, since there is no need to preprocess the sample, and any constituent in the sample can be visualized as long as there is information about the FF of the pure target constituent.

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Keywords: Fluorescence fingerprint; imaging; distribution; cosine similarity

1. Introduction

One of the important factors determining the palatability of bread is texture. The texture of the final

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product is affected by multiple processes in bread making, but it can be said that the first step, the mixing process, is very important in dough production [1]. The point when the dough is fully developed can be determined using conventional mixers, such as the Farinograph and Mixograph. However, the total amount of energy required to fully develop the dough to this point depends on many factors: the mixing speed, mixer design and flour characteristics [2], water content of the dough [3] and the quantity of other ingredients such as sugar [4] and shortening [5].

Consequently, many studies have attempted to directly define the condition of the dough at its optimally mixed state by performing rheological measurements and examining the structure behind it [6]. Many microscopy imaging techniques have been developed for this purpose.

The light microscopy and electron microscopy approaches are the main methods used to visualize the structure of dough. While light microscopy can be used to visualize specific chemical compounds in the complex mixture and is therefore suitable for foods that have complicated and multicomponent materials [7], it always involves the staining preprocess. The problem with staining is that the results could markedly vary depending on the selection of stains and staining conditions, such as the concentration, solvent and staining time. The researcher's technique to stain the sample evenly or prevent color degradation may also affect the result. Electron microscopy, such as scanning electron microscopy (SEM), is often used in the visualization of dough development because of its high magnification and clear images, but the risk of sample alteration is difficult to eliminate since the sample needs to be either dried or frozen below -80 ºC and covered with a 5-20-nm-thick metal layer [8]. Environmental scanning electron microscopy (ESEM) allows the sample to be observed without dehydration or surface coating [9], but differences between chemical compounds cannot be visualized.

To visualize the distributions of various constituents in food without any preprocessing, Tsuta et al. [10] developed an imaging technique using the fluorescence fingerprint (FF). The FF, also known as the excitation-emission matrix (EEM), is a set of fluorescence spectra acquired at consecutive excitation wavelengths to create a three-dimensional diagram. The pattern of this diagram, like a fingerprint, is unique for each constituent. The FF has an advantage over the conventional emission spectra because it includes emission bands excited at multiple excitation wavelengths, making it possible to observe slight differences in fluorescence characteristics [10]. Therefore, fine distinctions can be made between constituents, which would otherwise be indistinguishable.

Past studies have followed the structural changes in dough through the mixing process using stains [11] or SEM [12] and described the changes qualitatively. In this study, the FF imaging method was used to follow the changes in the same way, but aiming to make full use of the advantages of the method such as the few preprocesses needed or the sensitivity toward different constituents. Furthermore, it aimed to explain the changes more objectively than past studies by extracting parameters from the visualized images, which could actually be used to quantify the distribution and structure of the dough.

2. Materials and Methods

2.1. FF imaging system

An FF imaging system consisting mainly of a Xenon light source (MAX-302, Asahi Spectra), a monochrome charged coupled device (CCD) camera (ORCA-ER-1394, Hamamatsu Photonics) and two sets of band-pass filters (HQBP filter and M.C. filter, Asahi Spectra) was constructed. This imaging system acquires the fluorescence image of the sample at consecutive excitation and emission wavelengths in the following procedure.

The light from the Xenon light source is filtered through the first band-pass filter and an excitation light of a specific wavelength is acquired. This excitation light is irradiated on the sample, causing the sample to emit fluorescence. The fluorescence image is taken in through an objective lens, passes through another band-pass filter which defines the emission wavelength and is captured with the CCD camera.

The band-pass filters at both ends of the imaging system are changed and the fluorescence image of the sample in the next set of excitation and emission wavelengths is acquired. This procedure is repeated until all the combinations of excitation and emission wavelengths which correspond to the wavelengths that make up the FF are used.

In the acquired data (hereafter referred to as the spatial FF data), each fluorescence image contains the fluorescence intensity emitted from an area in the sample corresponding to each pixel. Therefore, the whole set of fluorescence images contain the FFs of each pixel and since the FF contour pattern reflects the constituents of the measured sample, it is possible to visualize the distribution of specific constituents in the sample by analyzing the spatial FF data.

2.2. Sample Preparation

Flour (Camellia, Nisshin Flour Milling Inc.) and tap water (68% of flour weight) were mixed in a mixer (HPI-20M, Kanto Kongoki Industrial Co., Ltd.) to make bread dough. The dough was mixed for 1 min at 136 rpm to create "under-mixed dough", another 1 min at 136 rpm, 4 min at 248 rpm and 2 min 30 sec at 310 rpm to create "optimum-mixed dough", and another 7 min at 310 rpm to create "over-mixed dough". Approximately 100 g of dough was sampled at each stage. The dough sample was cut into small pieces of approximately 2 cm square and frozen at -80 ºC.

Pure gluten and starch which would be used as reference data when analyzing the FF of bread dough were extracted from another batch of dough. The dough was carefully kneaded in water to separate gluten from starch granules and other soluble substances. When the starch granules were washed away, the remaining gluten aggregated into a highly elastic mass that was then cut into pieces of approximately 2 cm square, frozen and kept at -80 ºC. The aqueous mixture containing starch and other soluble substances were centrifuged for 20 min at 7,000 rpm with a centrifugal separator (Himac CR21GIII, Hitachi Koki Co., Ltd.) set at 5 ºC. The lower layer, referred to as the primary starch was carefully separated, freeze-dried with a lyophilizer (Eyela FDU-830, Tokyo Rikakikai) and kept at -20 ºC.

The frozen dough and gluten were broken into small pieces approximately 0.5 cm square with a hammer and quickly embedded in a freeze embedding agent (Tissue-Tek O.C.T. compound, Sakura Finetek Japan) before the sample melted. The sample was frozen at -80 ºC until the O.C.T. compound was completely fixed, transferred to a cryomicrotome (Leica CM1850, Leica Microsystems Japan) cooled to -20 ºC and sliced to obtain sections of 20 μm thickness. The thin sections were mounted on a glass slide (Matsunami Micro Slide Glass) and left to dry completely at room temperature.

The freeze-dried starch was mixed with 80% distilled water to prepare an easily handled paste and was embedded in O.C.T. compound. The sample was frozen, sliced using a cryomicrotome and dried, following the method for gluten and dough. However, the thin sections of starch were sliced to be of 15 μm thickness, which enabled the clear observation of individual starch granules.

2.3. Fluorescence Image Acquisition

Data analysis and image processing were carried out using versatile numerical analysis software (MATLAB R2010a, The MathWorks, Inc.) and an image processing software (ImagePro ver. 6.3.0.424, Media Cybernetics).

The data was analyzed based on the Spectral Angle Mapper (SAM) algorithm, which calculates the "angle" between the FF of each sample (in this case, each pixel of the bread sample image) and that of the target constituent (in this case, gluten or starch). If the angel between the FFs of the sample and gluten was small, it would indicate that the two FFs were similar and this would suggest a high proportion of gluten in that area. The likewise could be said with starch. The angle was calculated from the inner product of the two FFs, and is defined by using equation *(1)*.

$$\vartheta = \cos^{-1}(X \cdot Y/(|X| * |Y|))$$
$$X \cdot Y = x_1 * y_1 + x_2 * y_2 + \cdots + x_n * y_n \qquad (1)$$
$$|X| = \sqrt{(x_1 * x_1 + x_2 * x_2 + \cdots + x_n * x_n)}$$

The variables and are the fluorescence intensities of the sample and the target constituent, respectively, under consecutive wavelength conditions (n=63). The target constituent in this case is gluten or starch. The angular value reflects the differences in the direction of the FF vector and takes values between 0 (exactly the same) and π(exactly the opposite).

The angular values were then converted into color indices through a color scale, and a pseudocolor image was constructed. The angular value to gluten was converted into shades of red, whereas the angular value to starch was converted into shades of green. In this way, a color was assigned to every pixel in the dough according to the calculated angular value and the corresponding color axis. The images showing the angular values to gluten and starch were combined to obtain a pseudocolor image.

Small air bubbles existed in the samples and these appeared as holes when the samples were sliced and made into thin sections. These bubble areas were masked based on linear discrimination analysis with the absolute value or the Euclidean norm of the FF vector as the variable for discrimination. Areas that were clearly inside the bubble areas or inside the sample areas were selected from all fluorescence images to create the training set from which the discriminant function was calculated. Using this discriminant function, every pixel of the image was classified into the “bubble area” or the “sample area”. The pixels classified to be in the bubble area were masked in black.

3. Results and Discussion

3.1. Pseudocolor image of dough

Fig. 1 shows the pseudocolor images of dough at the three mixing stages, under, optimum and over, colored according to the angular value between the FF of each pixel and the average FFs of gluten and starch. The areas in red and green are the areas with high proportions of gluten and starch, respectively. It can be seen that the gluten and starch distributions are heterogeneous in the under-mixing stage, but become more homogeneous or more “even” in the optimum- and over-mixing stages. This evenness of gluten and starch does not seem to change much from the optimum-mixing stage to the over-mixing stage.

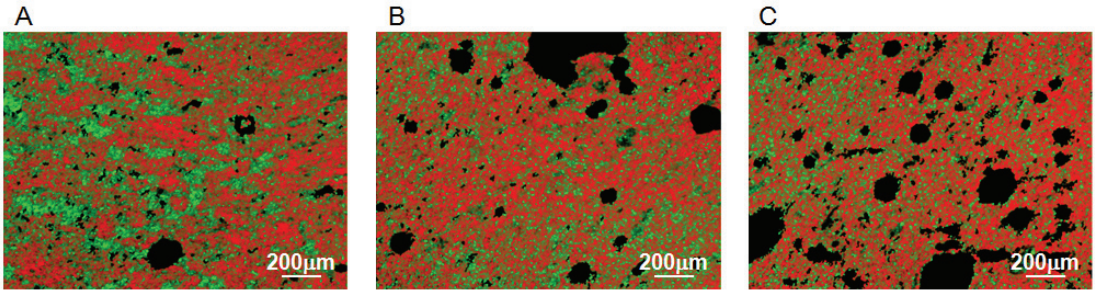

Fig. 1. Pseudo color images of dough in (A) under-mixing, (B) optimum-mixing and (C) over-mixing stages

The over-mixing stage is distinctive in the sense that the area of bubbles (the areas shown in black) is much larger than that in the other stages. Many large bubbles can be observed, some with a diameter of

over 200 μm. The dough in the under-mixing stage contains few of these big, circular bubbles, and the black areas that exist mainly on the border between the starch and gluten are small and of irregular shape. These observations should be confirmed by extracting quantitative parameters from the pseudo color images.

3.2. Quantitative analysis of gluten and starch distribution

To evaluate and quantify the distributions of gluten and starch and the differences between each mixing stage, a parameter concerning the evenness of the distribution was extracted. This parameter was calculated with the following algorithm. First, each image was divided into squares (number of squares = M) whose length and width was k pixels (k = 1, 2,…), and the ratio between the sum of angular value to gluten and starch was calculated for each square. The ratio for the mth square (m = 1, 2,…, M) was calculated using equation *(2)*.

$$ratio_m = \frac{\sum_{i=1}^{k^2} \theta_{mi\_glu}}{\sum_{i=1}^{k^2} \theta_{mi\_str}} \qquad (2)$$

θ_{mi_str} and θ_{mi_glu} are the angular values between the FF of the ith pixel (i = 1, 2,…, k^2) inside the mth square and the FFs of starch and gluten, respectively. Secondly, the standard deviation of $ratio_m$ was calculated. As shown in Fig. 2, in an image where the distributions of gluten and starch are uneven (A), the ratio between gluten and starch varies within each square, i.e., the standard deviation of $ratio_m$ shows a large value. Conversely, if the distributions of gluten and starch are fairly even (B), the standard deviation of $ratio_m$ becomes smaller. As the size of each square becomes larger (k takes a larger value), the standard deviation should become smaller.

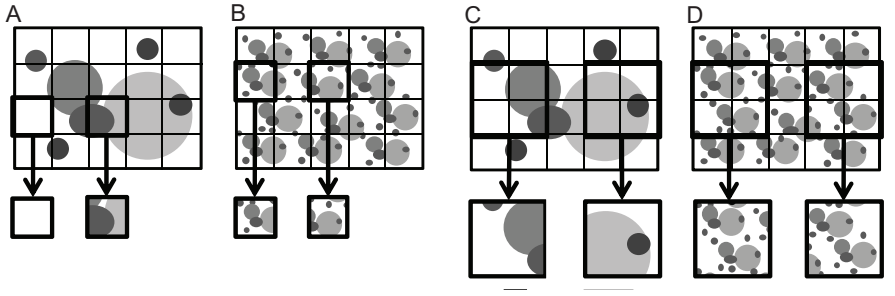

Fig. 2. Evaluation method of the evenness of gluten and starch distribution

Fig. 3 shows a graph explaining the evenness of gluten and starch in the under-, optimum- and over-mixing stages. As the length of the square increases, the standard deviation of the ratio between gluten and starch present in the square decreases, but the standard deviation is always higher in the under-mixed dough, i.e., the distribution is more uneven. There is no significant difference in the standard deviation for the dough between the optimum and over-mixing stages, showing that the evenness of the distributions of gluten and starch does not change after the optimum-mixing stage.

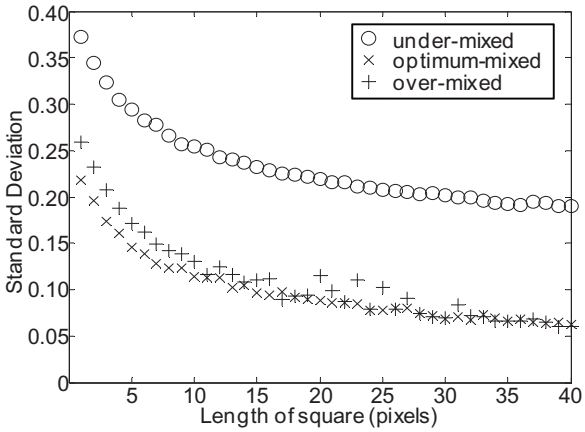

Fig. 3. Evenness of gluten and starch in the under-, optimum- and over-mixing stages

3.3. Quantitative analysis of bubble properties

The properties of the bubble areas were evaluated by the total bubble area, which is the total number of pixels classified as "bubble area". Fig. 4 shows the ratio of total bubble area to the total image area. The values for each sample are shown in the bar graph and the average value for each mixing stage is shown with an asterisk.

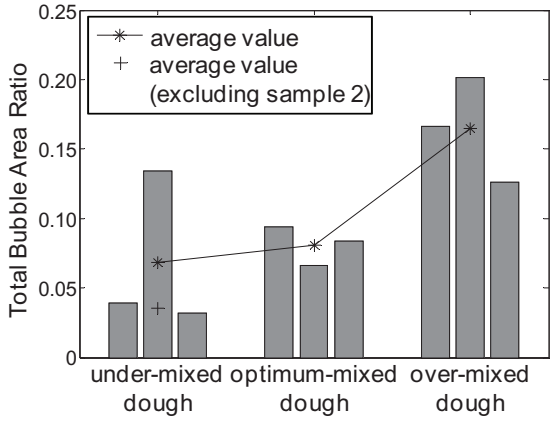

Fig. 4. Ratio of total bubble area to total image area

An obvious aspect in the under-mixing stage is that the second sample shows a much higher bubble ratio than the other two samples. This may indicate that the condition of the dough in the under-mixing stage varies markedly from place to place and that more mixing is needed to produce dough in which the bubbles are distributed evenly.

The average bubble ratio of the other two samples was calculated and shown in the graph with a "+" sign. If the second sample were to be excluded, it could be said that the area of bubbles increases significantly from the under-mixing stage to the optimum-mixing stage, supporting previous studies showing that one of the main functions of mixing is the incorporation of air nuclei [5]. Although more

sampling may be needed to actually prove that the second sample is an outlier and to confirm the increase in bubble area from the under-mixing stage to the optimum-mixing stage, the method itself is sufficient as a means of performing quantitative analysis.

The bubble ratio increases significantly from the optimum-mixing stage to the over-mixing stage, conforming to the observation from the pseudocolor image. As a whole, it could be concluded that the mixing procedure not only homogenizes the multiple constituents in the dough, but continuously brings air into it.

4. Conclusions

The FF imaging method developed in this study requires little preprocessing for the constituents in the sample to be visualized, compared with methods that require staining or freeze drying. This is particularly important for samples such as bread dough, which could easily deform or be modified. At the same time, the method proved to be sufficient for explaining the main changes occurring in the dough with mixing, such as the distributions of gluten and starch and the incorporation of air.

Many studies have examined the dough through multiple mixing stages, but the observations were mainly used to support other data such as rheological measurements or chemical analysis. Therefore, the observations were mainly qualitative and inherently subjective. In this study, quantitative parameters were extracted from the images to support primary observations or to acquire further information about the dough structure, which could not be perceived by just examining the images. Simple parameters such as the ratio of gluten to starch or the area of bubbles were chosen, but there is a possibility of extracting more complex parameters with morphological operations, for example.

This study has shown that two different phenomena progress when dough is mixed, the distributions of gluten and starch become more “even” as the dough approaches the optimally mixed condition and excessive air is incorporated as the dough goes beyond it. Therefore, optimally mixed dough could be defined as a condition where the gluten and starch are homogeneously distributed, while there is no excessive air incorporated in the dough.

Actually, there are many more changes taking place during the mixing process, which could be revealed with the visualization of other constituents and the quantification of its distributions. Any constituent in the sample can be visualized by the FF imaging method as long as there is information about the FF of the pure target constituent. Furthermore, the visualized image in digital form allows the smooth application of quantification methods. This versatility may also be considered as a big advantage of the FF imaging method.

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